# Differentially expressed genes in visceral or subcutaneous adipose tissue of obese men and women

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**Abstract** **There is growing evidence that the distribution of adipose tissue in the body is of importance in the development of metabolic complications of obesity, such as diabetes, hypertension, and hyperlipidemia. The aim of this study was to identify differentially expressed genes in subcutaneous and omental human adipose tissue in obese men, using a subtractive hybridization strategy. From the obtained set of differentially expressed transcripts, we also aimed to identify genes that have a sex-specific pattern of expression in omental or subcutaneous adipose tissue. Representational difference analysis (RDA) was performed on cDNA from subcutaneous and omental fat tissue from a man with extreme abdominal obesity. Forty-four putatively differentially expressed genes were identified. The obtained RDA products were spotted onto glass slides to screen for differential expression in other obese patients by using a microarray hybridization procedure. Five genes were confirmed to be differentially expressed in subcutaneous or omental adipose tissue from male or female obese patients. One gene was detected only in males and was found to be upregulated in subcutaneous tissue. The findings extend previous knowledge that different fat depots have differential gene expression and indicate that sex differences exist in adipose gene expression patterns.** Linder, K., P. Arner, A. Flores-Morales, P. Tollet-Egnell, and G. Norstedt. **Differentially expressed genes in visceral or subcutaneous adipose tissue of obese men and women.** ***J. Lipid Res.*** **2004. 45: 148–154.**

**Supplementary key words** representational difference analysis • differential gene expression • DNA microarray analysis • adipsin • ras • phospholipid transfer • calcyclin

It is well established that accumulation of visceral fat is associated with a higher risk for development of obesity-related diseases such as type 2 diabetes, cardiovascular disease, hypertension, and hyperlipidemia (1). Adipose tissue distribution differs between men and women, and visceral obesity is much more common among men than women (2, 3). The metabolic and endocrine functions of adipose tissue from various depots differ in a way that may explain the association of visceral but not subcutaneous fat with obesity-related cardiovascular and metabolic problems (4).

Regarding the metabolic function of fat, visceral adipose tissue is more sensitive to the stimulation of lipolysis by catecholamines, whereas subcutaneous fat is more sensitive to the antilipolytic effects of insulin. Concerning endocrine function, visceral and subcutaneous adipocytes have different capacities to produce hormones and enzymes. Depot-related variation in mRNA expression has been shown for several genes, including leptin, TNF-α, angiotensinogen, PAI-1 (4), and recently, carboxypeptidase E and thrombospondin-1 (5).

The mechanisms responsible for depot differences in adipose function are unknown. It is possible that fat cells in various regions have different origins and, because of this, express different genes. Recent indirect evidence supports this idea, because newly formed adipocytes in human subcutaneous and visceral fat were shown to maintain the phenotypic site differences of mature adipocytes (6).

The major aim of the present study was to determine differences in gene expression patterns between subcutaneous and omental adipose tissue. We have used representational difference analysis (RDA) and microarrays to identify depot-specific genes that might explain the different metabolic and endocrine actions of these tissues, and possibly their different roles in the development of obesity-related diseases. Using RDA in two directions, i.e., with cDNA derived from subcutaneous adipose tissue subtracted from omental adipose tissue and the other way around, we cloned 44 cDNA fragments from a man with extreme abdominal obesity. We further used these 44 cDNA fragments to test differential expression in 6 other obese males and in 11 female patients using microarray hybridization. Combining RDA with microarray analysis enabled high throughput screening of the differentially

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cloned products. This procedure led to the identification of five genes with depot-specific expression in either males alone or in both sexes.

## MATERIALS AND METHODS

### Subjects

The obese subjects (7 males and 11 females) included were members of a subgroup of patients participating in a clinical trial of adjustable gastric banding (7). From these subjects, it was possible to obtain fat specimens from omental as well as subcutaneous adipose tissue (the latter was taken from the surgical incision). All subjects were Caucasian and born in Sweden. Except for obesity, they were healthy and did not use any regular medication. Clinical data are recorded in **Table 1**. The study was approved by the Ethics Committee of Karolinska Institute, Stockholm. All individuals gave informed consent to participate in the study. The patients fasted from 10 PM the day before surgery, and only saline was given intravenously before adipose tissue was removed, which took place at the beginning of surgery. A tissue specimen (~10 g) was taken from the abdominal surgical incision (subcutaneous fat) and from the major omentum. Premedication and general anesthesia were given as described (7). The specimen was immediately frozen in liquid nitrogen and stored at −70°C. Subcutaneous and omental adipose tissue from one male patient with extreme abdominal obesity was chosen for the procedure of subtractive cDNA hybridization using RDA (8). This patient was selected from 16 of the above-mentioned patients who had undergone an abdominal computerized tomography (CT) scan prior to surgery (the remaining two subjects could not be investigated by CT for technical reasons). Intra-abdominal and subcutaneous fat areas were determined at vertebrae L4-L5. We selected the subject that had the greatest intra-abdominal:subcutaneous adipose area ratio. He was 57 years of age and had a body mass index of 39 kg/m$^2$. His proportion of visceral versus total adipose area was 53% (426 cm$^2$ visceral fat area, 352 cm$^2$ subcutaneous fat area) and waist-to-hip ratio was 1.10. In attempts to verify differential gene expression, we used a pool of patient samples to minimize biological noise. Adipose tissue from 6 males and 11 females was used to prepare female or male, or visceral or subcutaneous RNA pools, respectively.

### RNA preparation

Subcutaneous and omental adipose tissue from individual subjects was homogenized, and total RNA was isolated using Trizol reagent (Life Technologies, Inc., Grand Island, NY) according to the protocol supplied by the manufacturer. The quality of the RNA samples was ascertained on denaturing agarose gels, and the concentration was determined spectrophotometrically. For verification of RDA results, four RNA pools were prepared. Equal amounts of total RNA were mixed to generate omental and subcutaneous RNA pools, using starting material from 6 males or 11 females.

TABLE 1. Clinical characteristics

| Measure | Males | Females | $P$ |
|---|---|---|---|
| n | 7 | 11 | |
| Age, years | 46 ± 1 | 40 ± 11 | 0.87 |
| Body mass index, kg/m$^2$ | 41 ± 2 | 43 ± 1 | 0.46 |
| Waist-to-hip ratio | 1.05 ± 0.04 | 0.91 ± 0.03 | 0.005 |
| PL-glucose, mmol/l | 6.1 ± 2.3 | 6.2 ± 2.5 | 0.96 |
| PL-insulin, mU/l | 22.6 ± 13.6 | 18.6 ± 9.5 | 0.47 |
| PL-cholesterol, mmol/l | 6.4 ± 0.9 | 5.4 ± 1.0 | 0.05 |
| PL-HDL-cholesterol, mmol/l | 1.1 ± 0.1 | 1.2 ± 0.1 | 0.85 |
| PL-triglycerides | 2.5 ± 1.1 | 1.8 ± 0.9 | 0.15 |

PL, plasma. Values are mean ± SD; comparisons were by Student's unpaired $t$ test.

### cDNA RDA

cDNA RDA was performed as previously described (8). One hundred thirty micrograms of total RNA from subcutaneous or omental fat tissue, respectively, was used to enrich poly(A)+ RNA (mRNA) using oligo-(deoxythymidine) paramagnetic beads (Dynal AS, Oslo, Norway). cDNA was synthesized using a kit purchased from Promega Corp. (Madison, WI).

cDNA obtained from subcutaneous and omental adipose tissue from the chosen male subject was used as driver (subcutaneous) and tester (omental), or vice versa, to generate gene products (representations) that were induced or repressed in omental adipose tissue. After two rounds of subtraction and amplification, using tester:driver ratios of 1:100 and 1:800, difference products (DP2) were visualized on a 2% agarose gel. After being excised and eluted from the gel, seven bands were cloned into the *Bam*H1 site of the pBluescript II SK+ vector (Stratagene, La Jolla, CA). Between 96 and 140 isolated bacterial colonies were picked from each excised gel slice and grown overnight in 1.5 ml Luria-Bertani medium. Plasmid minipreparations were made using the Wizard system (Promega Corp.).

### Sequence analysis and functional annotation

Sequence analysis of differentially expressed cDNA products, in total 768 clones, was performed using cycle sequencing with dye-labeled nucleotides (Big-Dye, Perkin-Elmer Corp., Norwalk, CT) loaded on a PE Applied Biosystems 377 DNA sequencer (Perkin-Elmer Corp.). Redundancy analysis and vector clipping were performed using Staden package data processing programs (9). The sequences were analyzed for homologies with published sequences in the nonredundant and expressed sequence tag (EST) divisions of the public databases of the National Center for Biotechnology Information using the Blast N/X software (10). Clones with more than one gene sequence were discarded. Only sequences longer than 50 bp with more than 96% homology to known human genes or ESTs were accepted for annotation. Sequences with no hit were also tested for annotation in the Celera database. Functional prediction was performed by using the information at Unigene (http://www.ncbi.nlm.nih.gov/Unigene/), Online Mendelian Inheritance in Man (OMIM), Locuslink, and Medline databases.

### Generation of micro array chip with the cloned RDA products

Minipreparations of the cloned RDA products were further PCR amplified using vector-specific primers T3 and T7. The amplified inserts, each produced by two pooled 100 μl PCR reactions, were purified by ethanol precipitation and resuspended in 40 μl 3 × SSC, and 1 μl 1% Sarcosyl was added to each clone. Each PCR product was checked on an agarose gel. Products that showed double bands were omitted from further evaluation. The amplified RDA products were printed on CMT GAPS amino silane-coated slides (Corning, Inc., New York) using a GMS 417 arrayer (Genetic Microsystems, Woburn, MA) with four pins. To ensure reproducibility of hybridization, each RDA clone was spotted in triplicate at different locations on the chip. Two housekeeping genes, β-actin and GAPDH, were added as normalization controls to allow comparisons between chips. Leptin cDNA was also printed as an external control. After printing, the slides were postprocessed as described previously (11) and stored in a dark, dust-free box until hybridization. Before hybrid-

ization, the slides were prehybridized in hybridization buffer (5 × SSC, 0.1% SDS, 1% BSA) at 42°C for 90 min.

### Labeling and hybridization of RNA to the cDNA microarray

The protocol employed for probe labeling and purification was essentially as described previously (11). Twenty micrograms of total RNA from the subcutaneous and omental female or male pool was used to generate fluorescently labeled cDNA probes. The labeling procedure was repeated once, and dye swap was performed to avoid differences in labeling efficacy. Fluorescently labeled cDNA probes were synthesized by oligo-dT-primed reverse transcription reaction using Superscript II (Life Technologies, Inc.) in the presence of cytochrome-labeled nucleotides (Cy3- or Cy5-conjugated uridine 5′ triphosphate, New England Nuclear) (11). Cy3- and Cy5-labeled cDNA probes were combined and purified using Microcon 30 (Millipore). The final volume was adjusted to 15 μl, with hybridization buffer consisting of 5 × SSC, 0.2% SDS, 10 μg poly(A) RNA, and 10 μg yeast tRNA. After heating at 100°C for 2 min, the probes were added to the array and covered with a 22 × 22 mm cover slip (Grace Bio-Labs, Bend, OR). The array chip was placed in a sealed hybridization chamber (Corning), and the hybridization took place at 65°C for 15–18 h. The array was then washed (11) and immediately scanned using a GMS 418 scanner (Affymetrix, Santa Clara, CA). The hybridization was repeated once for each comparison with inversely labeled probes.

### cDNA microarray image analysis

The Cy3 and Cy5 images were superimposed and analyzed using GenePix Pro software (Axon Instruments, Union City, CA). The net fluorescent signal at each spot from Cy5 and Cy3 dyes was subsequently compared. Automatic and manual flagging were used to localize absent or very weak spots that were excluded from analysis. The signal from each spot was calculated as the average intensity of the spot minus the background. As a criterion for distinguishing a signal from noise, we used a signal that was greater than 1.4 times the background (12). For normalization between chips, we used the housekeeping gene GAPDH, recently thoroughly examined and recommended as a robust housekeeping gene in adipocyte studies (13). Only transcripts that had a Cy5:Cy3 ratio higher than 1.4 times the background in at least two of three spots (with the same cDNA) were used for further analysis. The cutoff for selection of upregulated and downregulated genes was set to 1.5 (14, 15). Only changes in gene expression that were reproduced in both hybridizations are presented.

### Statistical analysis

Values are mean ± SD. When men and women were compared, Student's unpaired *t*-test was employed. Concerning chip analysis, see previous section.

## RESULTS

In the present study, we have used RDA and cDNA microarray screening to identify genes that might be important in the development of intra-abdominal obesity. The aim was to find novel genes differentially expressed in subcutaneous and visceral adipose tissue and also to investigate whether these genes differed in a male and a female obese population. RDA is a sensitive and efficient PCR-based subtraction method enabling unbiased cloning of differentially expressed gene fragments. Repeated subtraction and amplification rounds yield an efficient depletion of ubiquitous gene fragments from both cDNA populations, thereby cloning the genes that are most differentially expressed.

### cDNA RDA

To search for differences in gene expression between subcutaneous and omental human adipose tissue that are most relevant for visceral obesity, cDNA RDA was performed on adipose tissue obtained from a man with extreme visceral obesity. This patient, who was selected from among subjects who had undergone abdominal CT, had the greatest intra-abdominal:subcutaneous adipose area ratio. Representations (digested and amplified cDNA), generated from subcutaneous and omental adipose tissue, respectively, were used as driver and tester, or vice versa, to generate differentially expressed transcripts. The RDA resulted in a reduction in complexity of the two cDNA populations ranging from the representations to the final DP2 products (**Fig. 1**). Whereas the representations show a smear of products ranging from 30 to 1,000 bp, DP2 has clearly visible bands from 50 to 500 bp in size. To subclone as many different gene products as possible, three bands from DP2-subcutaneous and four bands from DP2-omental were excised from the gel and subcloned individually. Several (384) clones from each library were picked and sequenced. After sequence alignments, using Staden package data processing programs (9), the sequences were analyzed for homologies with published sequences in the nonredundant and EST divisions of the public databases using the Blast N/X software (10). Out of 768 clones sequenced, 44 unique sequences fulfilled our criteria for selection (see Materials and Methods). They were annotated and functionally grouped according to Unigene, Locuslink, or OMIM. The Unigene cluster identities of the sequences and the functional groupings are shown in **Table 2**.

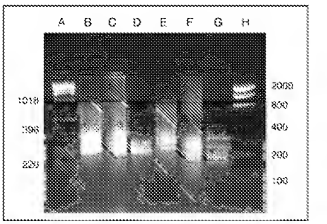


**Fig. 1.** Agarose gel electrophoresis of representations and difference products identified by cDNA representational difference analysis (RDA). Ethidium bromide-visible bands in a 2% agarose gel correspond to: (A and H) size markers; (B and E) representations of subcutaneous (B) or omental (E) adipose tissue; (C and F) difference product 1 (DP1) after one round of subtraction/amplification; (D and G) difference product 2 (DP2) after two rounds of subtraction/amplification. cDNA obtained from subcutaneous adipose tissue was used as a tester to generate DP1 and DP2 (C and D). Similarly, cDNA obtained from omental adipose tissue was used as a tester to generate DP1 and DP2 (F and G).

TABLE 2. Annotation and detection of cDNA clones obtained in RDA

| Annotation | Unigene Number | Adipose Expression Known | Detection by cDNA Microarray |
|---|---|---|---|
| Immune response | | | |
| Complement factor B, properdin | Hs.69771 | X | |
| Serum amyloid A1 β gene | Hs.332053 | X | X |
| Adipsin/complement factor D precursor | Hs.155597 | X | X |
| Lipid metabolism | | | |
| PLPT | Hs.283007 | X | X |
| Annexin A8 | Hs.87268 | | |
| Protein biosynthesis | | | |
| Ribosomal protein L37a | Hs.296299 | X | |
| 40S Ribosomal protein S3 | Hs.252259 | | |
| Ribosomal protein L28 | Hs.4437 | X | |
| Ribosomal protein L27 | Hs.111611 | | X |
| Eukaryotic translation initiation factor 3, subunit 9 | Hs.57783 | X | |
| Ribosomal protein, large | Hs.73742 | | X |
| Signal transduction | | | |
| Ras homolog gene family, member G | Hs.75082 | | X |
| Calcyclin, S100 calcium binding protein | Hs.275243 | X | X |
| Cell structure | | | |
| Cytokeratin 8 mRNA | Hs.242863 | | |
| Gelsolin | Hs.290070 | X | |
| Mesothelin | Hs.135981 | | |
| Actin, β | Hs.288061 | X | X |
| α-Catenin | Hs.178452 | | |
| Carbohydrate or amino acid metabolism/energy | | | |
| Glyceraldehyde-3-phosphate dehydrogenase | Hs.169476 | X | X |
| Glutamine synthetase | Hs.179171 | X | |
| ATP synthase, H+ transporting | Hs.155101 | | X |
| Acetyl-CoA synthetase 2 | Hs.14779 | | |
| Transaldolase 1 | Hs.77290 | | |
| Mitochondrial genes | | | |
| Mitochondrial genome, similar to cytochrome C oxidase | | | X |
| Mitochondrial genome | Hs.193709 | | X |
| Secreted proteins | | | |
| SPARC/osteonectin | Hs.111779 | X | |
| Unknown function | | | |
| SSN4 protein gene | Hs.193863 | | |
| Human chitinase (HUMTCHIT) | Hs.154138 | | |
| Hypothetical protein FLJ23142 | Hs.26099 | | X |
| EST | Hs.354402 | | X |
| Homo sapiens hypothetical protein FLJ20922 | Hs.50813 | | X |
| Homo sapiens upregulated by 1,25-dihydroxyvitamin D-3 | Hs.179526 | | |
| EST, hypothetical protein PRO2730 | Hs.194110 | | |
| Amplified in osteosarcoma (OS-9) | Hs.76228 | | |
| Homo sapiens 12q24 BAC RCPH11-449D10 | Not found in Unigene | | |
| Clone 39889 on chromosome 1p34.3-36.13 | Hs.179619 | | |
| EST (HUMSUPY457) | Hs.333195 | | |
| Chromosome X clone BWXD759 | Hs.194439 | | |
| Homo sapiens chromosome 5 clone CIT-HSPC_551A13 | Hs.194549 | | |
| Homo sapiens chromosome 19, cosmid F19847 | Hs.37062 | | |
| Human chromosome 14 DNA sequence: BAC R-1023I22 | Hs.14144 | | |
| Homo sapiens PAC clone RP5-892G19 from 7q31.3-q33 | Hs.194549 | | |
| Homo sapiens BAC clone CTA-356E1 from 7q11.23-q21.1 | Hs.131342 | | |
| Homo sapiens 12p13.3 PAC RPCI5-1096D14 | Hs.241427 | | X |

EST, expressed sequence tag; PLPT, phospholipid transfer; RDA, representational difference analysis; SPARC, secreted protein, acidic and rich in cysteine. Sequences were compared with homologies of published sequences in the nonredundant and EST divisions of the public databases using BLAST N/X software, and the accession number with the best match was mapped to the corresponding Unigene cluster. The transcripts were further categorized into functional groups according to Unigene, Locuslink, or Online Mendelian Inheritance in Man. Previously reported adipose expression according to Unigene is indicated. The detection column indicates whether the transcript was detected (i.e., had a Cy5/Cy3 ratio higher than 1.4 over the background in at least three of four independent hybridizations) in the microarray experiments examining a pool of female or male obese individuals.

### Verification of cloned adipose genes using cDNA microarray

To evaluate whether the RDA clones obtained could also mark gene expression differences in other obese patients, we screened the 44 different cDNA clones for differential expression using DNA microarrays. The obese subjects (6 males and 11 females) included were members of a subgroup of patients participating in a clinical trial of

TABLE 3. Screening results regarding gene expression differences in subcutaneous and omental fat in obese patients[a]

| | Unigene Number | Fold Difference Omental/Subcutaneous (SD) | |
|---|---|---|---|
| | | Female | Male |
| Higher in omental | | | |
| Ras homolog gene family, member G | Hs.75082 | 8.8(5.6) | 9.2(7.3) |
| Phospholipid transfer protein | Hs.283007 | 2.3(0.34) | 5.2(1.4) |
| | | Fold Difference Subcutaneous/Omental (SD) | |
| | | Female | Male |
| Higher in subcutaneous | | | |
| Calcyclin | Hs.18341 | 2.1(0.25) | 1.7(0.29) |
| Adipsin | Hs.17559 | 1.7(0.18) | 1.6(0.26) |
| PAC clone 12p13.3 | Hs.341427 | Not detected | 1.6(0.39) |

The changes reported are >1.5-fold. Values are the average of results in two different hybridizations. Each hybridization consists of triplicate measurements of each transcript. The variation of measurement in one chip experiment for these transcripts ranged between 5% and 10%.

[a] Verified as significantly upregulated and downregulated transcripts in subcutaneous versus omental adipose tissue using cDNA microarray analysis.

adjustable gastric banding (7). On average, the subjects were morbidly obese (body mass index >40 kg/m$^2$) (Table 1). The RDA-derived microarrays were hybridized with probes derived from subcutaneous and omental fat from pooled male or female RNA. As shown in Table 2, 16 (36%) of the 44 RDA clones were detected. Four clones were differentially expressed in the female pool and five in the male pool. These gene products are listed in **Table 3**. A schematic overview of the RDA and microarray experiments is shown in **Fig. 2**.

Leptin cDNA, although not among the RDA clones, was loaded on the chip as a positive control. Leptin cDNA gave a stronger signal in subcutaneous fat (1.9-fold induction), which confirms several previous findings [as reviewed in ref. (16)]. As an additional verification, mRNA levels of leptin were also determined by solution hybridization-RNase protection analysis (17), which showed 2.8× overexpression in the subcutaneous adipose (data not shown).

## DISCUSSION

The purpose of this study was to search for novel genes that may be linked to a disproportionate accumulation of adipose tissue in the abdominal region. Previous studies (18) have used a candidate gene approach to search for depot-specific adipose gene expression. We used RDA for differential screening, because it is a method that identifies genes that are expressed at different levels in one sample over the other in an unbiased manner. We chose to search for differential gene expression between omental and subcutaneous fat in a man with extreme visceral obesity, because we assumed that we would be most likely to find genes of interest in such a subject. Seven hundred sixty-eight cDNA fragments that, after sequence comparisons, could be deconvoluted to 44 nonredundant gene fragments were identified. Many of these had previously been found to be expressed in adipose tissue, e.g., adipsin; serum amyloid A; secreted protein, acidic and rich in cysteine (SPARC); phospholipid transfer (PLPT) protein; and several ribosomal proteins (19, 20). However, some had not previously been reported to be expressed in adipose tissue.

In differential cloning procedures, one may pick up false positives, i.e., genes that are not differentially expressed. In this study, we chose to evaluate this by testing a new set of male patients. In addition, we attempted to search for sex differences in expression by analyzing a group of obese females. To enable a rapid screening, we used cDNA microarray technology. The 44 RDA clones were spotted in triplicate onto glass slides and hybridized to fluorescently labeled cDNA from the above-mentioned male and female RNA pools.

Using the microarray technique, we could verify that RDA can select for differentially expressed genes. Thirty-

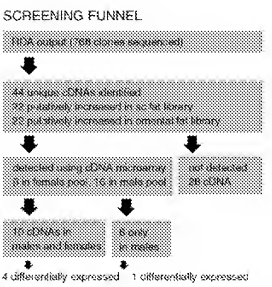


**Fig. 2.** A schematic overview of the RDA and microarray experiment. The complexity of the RDA output was reduced by sequence alignments and redundancy analysis, followed by biological and methodological validation using microarray analysis.

six percent of the RDA clones were detected by cDNA microarray, and 31% of these were differentially expressed according to the chosen criteria. The strategy of using potential differences in one patient as the basis for screening in another set of patients was chosen as a filter to search for common differences. The relatively low percentage of detected clones could indicate biological variation between the RDA patient and the pool, but methodological explanations are also plausible. The cDNA microarray technology has limitations in terms of sensitivity, and in studies using RDA in combination with cDNA microarray, one problem might be the cDNA probe length. The RDA DP2-product lengths are between 75 and 500 bp, and the optimal probe length for cDNA microarray is around 1,000 bp. cDNA-RDA followed by microarray validation has also been used in two recent studies on adipose tissue (21, 22). Both studies reported the same observation as described by us, i.e., many of the RDA clones were not detected with the microarray analysis, resulting in a relatively low percentage of detected transcripts. We could detect 36% of the RDA products, and 31% of these were differentially expressed according to chosen criteria. Using the same approach, Boeuf et al. (22) confirmed differential expression for 30% of obtained RDA clones with microarray screening.

It is clear that our choice to sequence 768 clones in this comparison did not fully reveal all differences. Concerning the cDNA-RDA technique, not all of the differentially expressed genes are necessarily enriched during the procedure. Lack of Sau 3A1 restriction sites in the mRNA may generate less than 100% coverage of expressed genes in the representations. Large representation fragments may not be efficiently amplified by PCR. Leptin, which has a well-known differential expression with overexpression in subcutaneous fat, was not among the sequenced cDNA-RDA-clones. Nevertheless, the expected site difference in leptin expression was found in the control clone on the cDNA microarray. The fact that many of the clones were found in only a single copy makes one suspect that picking and sequencing more clones would reveal more genes. The PCR amplification step in the RDA can increase small expression differences and enable detection of low-expressed transcript. The lower sensitivity of microarray might then fail to verify these transcripts. An alternative method including PCR amplification steps, such as real-time RT-PCR, might be more suitable, and the low RNA amounts needed could also enable analysis of individual patient samples.

Taking into account the above-mentioned methodological limitations, it seems likely that the true differences between subcutaneous and omental adipose tissue are underestimated in this study.

Although a certain caution is warranted, because our investigation is limited to mRNA expression (not protein) and the size of the biological sample is low, it is of interest to discuss the possible clinical relevance of our findings. Intra-abdominal obesity is usually associated with abnormalities in the lipoprotein profile. We observed an overexpression of PLPT mRNA in the omental fat in obese males and females. PLTP is a member of the lipid transfer, lipopolysaccharide binding protein family. It is a carrier protein that shuttles between lipoproteins to redistribute lipids and therefore has an important role in determining HDL levels. Expression of PLPT mRNA in human adipose tissue was demonstrated in 2000 by Duserre, Moulin, and Vidal (23). In agreement with the present study, they found a depot-related difference in mRNA levels, with overexpression of PLPT in omental adipose tissue in subjects with moderate obesity. The present study and previous studies (23) suggest that PLPT from adipose tissue (visceral, in particular) might contribute to plasma levels of PLPT. Plasma PLPT activity has been related to insulin resistance and to alterations in HDL metabolism in obese nondiabetic and obese type-2 diabetic patients (24).

A novel and interesting finding is the overexpression of calcyclin in subcutaneous adipose tissue of men and women. Calcyclin, or S100A6, is a calcium binding protein whose expression is upregulated in proliferating and differentiating cells (25). S100A6 belongs to a large family of $Ca^{2+}$ binding proteins that have been implicated in several human diseases, such as rheumatoid disease, acute inflammatory lesions, cardiomyopathies, and cancer (26). S100 protein content is induced in 3T3-L1 cells during differentiation to adipocytes and released when the cells are treated with lipolytic hormones (27). The release from fat cells is also stimulated by free fatty acids (27), and this stimulation can be inhibited by insulin (28).

We also found overexpression of adipsin in the subcutaneous adipose tissue in both males and females. This finding is consistent with observations reported by White et al. (29), including high expression of adipsin in subcutaneous adipose tissue. Human adipsin is identical to complement factor D. The complement system, consisting of ~20 proteins, plays an essential role in nonspecific and immunologically induced host defense. It is known that adipocytes are able to secrete the essential components of the alternative pathway: adipsin and factors B, C2, and C3 (22, 30, 31). Several of these factors (including adipsin) are precursors for adipose-derived acylation-stimulating protein, which is a key regulator of lipid turnover in human fat cells (32, 33).

Ras is a family of GTP binding proteins of the rho subfamily, which regulates organization of the actin cytoskeleton (34). The finding that ras G is differentially expressed, with overexpression in omental adipose of either sex, is interesting in light of the key signaling functions of ras.

As shown in Table 2, we cloned several ESTs of unknown function. Only one of them fulfilled our criteria for differential expression. Interestingly, it was detected and differentially expressed only in the male subcutaneous pool. The cDNA-assigned PAC clone 12p13.3 was not found in the public databases. Search performed in the Celera database showed no match against expressed transcripts. However, in the database section "human transcripts," which includes introns and nontranslated parts of genes, a 94% match against gene hCG24218 on chromosome 12 was found. This gene product codes for a calcium channel α2-δ3 subunit with accession number AJ272213.

At the present time, we are uncertain as to whether this clone represents a new splice variant, a genomic contamination, or an unspliced transcript.

In conclusion, we have used an unbiased method, RDA in combination with cDNA microarray screening, to identify genes with different expression levels in different fat depots. We report for the first time differential expression of calcyclin, ras, and adipsin between subcutaneous adipose tissue and omental adipose tissue, which might be of pathophysiological importance in development of visceral obesity. This approach also detected a gene fragment not previously described included in PAC clone 12p13.3, which might be a calcium channel that is overexpressed in subcutaneous adipose tissue of men but absent in women. Further work is needed to clarify the biological significance of the latter finding for development of visceral obesity in men.

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